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**Method for detecting metals.**

57

Metals such as  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Sm}^{3+}$ ,  $\text{Cd}^{2+}$  and lanthanoids such as  $\text{La}^{3+}$ ,  $\text{Tb}^{3+}$  and  $\text{Yb}^{3+}$  are detected by measuring the luminescence produced in contact with reproduced aequorin obtained by adding coelenterazine or its analogues to an enzyme of aequorin (apoaequorin).

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1    METHOD FOR DETECTING METALS

          This invention relates to a method for detecting metals and especially to such a method in which a photo-  
5    protein is used, namely an enzyme of aequorin (apoaequorin), together with a substrate, as an emitter, namely coelenterazine or its analogues.

          Aequorin is a photoprotein obtainable from luminescent jellyfish which grow in the northern part of  
10   the west coast of America. When one molecule of aequorin is specifically bound to two or three molecules of  $\text{Ca}^{2+}$ , it oxidises the coelenterazine present, which acts as an emitter to produce luminescence. Thus, natural aequorin exists in the package state containing the enzyme  
15   (apoaequorin), the substrate (coelenterazine) and molecular oxygen ( $\text{O}_2$ ). When  $\text{Ca}^{2+}$  is added to natural aequorin, its reaction with the  $\text{Ca}^{2+}$ -binding sites present causes the coelenterazine to be oxidised, so that oxycoelenterazine,  $\text{CO}_2$  and  $h\nu$  (emission) are produced. This light emission  
20   can be detected by a photomultiplier and its sensitivity is so high that trace concentrations of  $\text{Ca}^{2+}$  of about  $10^{-7}\text{M}$  are measurable.

          The habitat of the jellyfish which produce natural aequorin is limited to the aforesaid coastal  
25   region and the season in which they are produced is also

1 limited. The yield of natural aequorin is only about  
10 mg per 50,000 jellyfish. It is thus very important to  
obtain aequorin, but a certain supply cannot be assured.

5 The inventor of the present invention has cloned  
the cDNA for aequorin obtained from these jellyfish by a  
recombinant DNA method. This cDNA clone was named plasmid  
pAQ440 (Japanese Patent Application 59-176125). Then, a  
plasmid containing a promoter in which the pAQ440 gene was  
10 inserted was transformed in a bacterium and it was found  
that the natural and fused type of aequorin (apoaequorin)  
could be efficiently expressed in E.coli (Japanese Patent  
Application 60-280259). This method provided a stable  
supply of aequorin.

15 The present invention further improves the  
method for detecting  $\text{Ca}^{2+}$  concentrations using natural  
aequorin and also provides a method for detecting metals,  
such as  $\text{Ca}^{2+}$  and others, using apoaequorin biosynthesised  
in E.coli.

20 According to one aspect of the present invention,  
a method for detecting metals is provided, wherein an  
enzyme of aequorin (apoaequorin) produced by biosynthesis  
in E.coli is used and wherein a substrate employed is  
coelenterazine or its analogues.

25 According to another aspect of this invention, a  
recombinant DNA molecule is characterised by comprising  
biologically pure aequorin synthesised by reacting  
coelenterazine or its analogues with apoaequorin.

30 Preferably, in carrying out the method of the  
invention, the aequorin enzyme (apoaequorin) is produced  
using the aequorin cDNA obtained by the biosynthesis  
method disclosed in the above-mentioned patent application  
and then, by adding coelenterazine as a substrate to the  
enzyme, aequorin having the same properties as natural  
aequorin is obtained. The luminescence shown by various  
35 metals when reacted with this synthesised aequorin is

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1 measured, in order to detect and/or measure the amount of  
such metals. As a result, the presence of  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  
 $\text{Sm}^{3+}$  can be observed. For  $\text{Cd}^{2+}$ , its presence can be  
confirmed from the inhibitory rate of luminescence of  $\text{Ca}^{2+}$ .  
5 Lanthanoids such as  $\text{La}^{3+}$ ,  $\text{Tb}^{3+}$  and  $\text{Yb}^{3+}$  also can be  
detected.

The following description serves to illustrate  
the invention more specifically.

(1) A method for producing aequorin (apoaequorin) in  
10 E.coli.

(1) Insertion of aequorin genes into an expression vector  
having a promoter.

As the expression vector into which the aequorin  
gene is inserted, the vector in which the promoter is  
15 cloned is used. As the expression vector, pUC9 can be  
cited as an example. As the promoter, lac, tac or trp  
derived from E.coli or PL of the  $\lambda$  phage can be used, by  
way of example.

Firstly, pDR540 having the promoter is digested  
20 by means of the restriction enzyme BamHI-HindIII. The  
resulting fragment containing the promoter is then separated  
and extracted by an electrophoresis method, e.g. in 8%  
acrylamide gel. By using the promoter thus extracted, the  
expression vector (piC9) can be obtained by cloning at the  
25 BamHI-HindIII site of the vector.

Then, after digesting this expression vector  
with HindIII, its terminals are repaired by E.coli DNA  
polymerase (Klenow fragment) in the presence of dATP,  
dGTP, dCTP and dTTP, both the terminals are ligated with  $T_4$   
30 ligase and an expression vector, piC10, is thus obtained.

In preparing to bind the promoter in the piC10  
with the aequorin cDNA gene, a synthetic nucleotide as a  
linker is cloned to piC10. Thus, the synthetic nucleotide  
linkers AR(5'GATCGATGGTCA-3') and AQ(5'AGCTTGACCATC-3')  
35 are first prepared, by a known synthetic method, and then  
annealed, after which phosphorylation of their 5'-terminals

1 is carried out using T<sub>4</sub> nucleotide kinase in the presence  
of ATP. The nucleotides thus phosphorylated are sub-  
sequently cloned, so as to construct plasmids in the form  
of repetition units of the linker at the BamHI-EcoRI site  
5 of the piC10, which has been constructed as mentioned  
above, and a recombinant DNA piC11 is so obtained.

Next, a HindIII-EcoRI fragment is separated from  
aequorin cDNA clone pAQ440 (see Japanese Patent Application  
59-176125), this fragment is then inserted into the  
10 HindIII-EcoRI site of the above piC11, and the desired  
piQ5, namely bacteria containing a promoter in which the  
apoaequorin gene has been transformed, is obtained. This  
piQ5 has the ability to produce aequorin proteins of  
natural type.

15 (2) Insertion of aequorin genes into an expression vector  
(pUC9, 9-1, 9-2) having a lac promoter in order to produce  
aequorin proteins of a fused type.

In a similar manner to that described in (1)  
above, PstI-EcoRI and HindIII-EcoRI fragments are separated  
20 from the cDNA clone pAQ440 and purified. Further, ex-  
pression vectors pUC9, 9-1, 9-2 having a lac promoter  
prepared by a similar method to that described in (1)  
above are obtained. Each of these fragments is cloned at  
the restriction enzyme site of each of these vectors to  
25 produce piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE. All of these  
piQ plasmids are under the control of the lac promoter and  
thus comprise aequorin proteins of the fused type having  
8-amino acid residues at an N-terminus.

(3) Production of proteins having aequorin activity using  
30 E.coli.

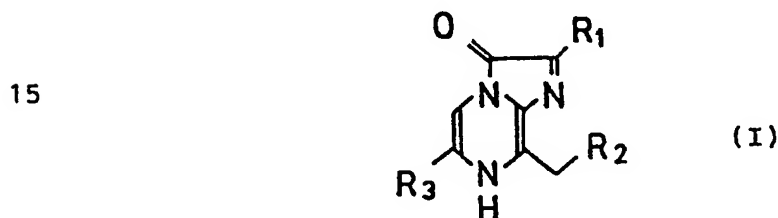
Each plasmid cloned to the expression vectors  
obtained by the methods of (1) and (2) is transformed in a  
strain, such as E.coli (D 1210), and aequorin proteins are  
produced. In this method, for example, E.coli strains  
35 containing plasmids are added to a certain volume of LB

1    broth containing a given concentration of ampicillin and  
 the E.coli strains are cultured. Subsequently, an ex-  
 pression-inducible reagent is added to the culture medium  
 and incubation is continued. The resulting culture medium  
 5    is separated by centrifugation and the resulting cells are  
 collected and washed with water.

(2) A method for synthesising coelenterazine and its  
 analogues.

10    In this method, coelenterazine obtained by a  
 known method can be used.

The coelenterazine used here has the following  
 general formula:



20    Coelenterazine of the natural type is represented  
 by the formula wherein  $R_1$  is  $p\text{-CH}_2\text{C}_6\text{H}_4\text{OH}$ ,  $R_2$  is  $\text{-C}_6\text{H}_5$  and  $R_3$   
 is  $p\text{-C}_6\text{H}_4\text{OH}$ .

Coelenterazine compounds represented by the  
 formula (I) can be obtained by a known method, for example,  
 the method disclosed by Inoue et al. (Published by Japan  
 25    Chemical Society, Chemistry Letters, 141-144, 1975).

Further, as analogues of coelenterazine, analogue  
 1, represented by the formula (I) wherein  $R_1$  is  $\text{-CH}_2\text{C}_6\text{H}_5$ ,  
 $R_2$  is  $\text{-C}_6\text{H}_5$  and  $R_3$  is  $p\text{-C}_6\text{H}_4\text{OH}$ , or analogue 2, represented  
 by the formula (I) wherein  $R_1$  is  $\text{-CH}_3$ ,  $R_2$  is  $\text{-C}_6\text{H}_5$  and  $R_3$   
 30    is  $p\text{-C}_6\text{H}_4\text{OH}$ , can be used. These analogues can be obtained  
 by a known method, for example, the method disclosed by  
 Halt et al. (Biochemistry, 18, (11) 2204-2210, 1979).

(3) A method for detecting metals.

35    Metals are detected by the following method, for  
 example.

1           The enzyme solution obtained by extraction from  
2           E.coli using the above process (3) is used. After dissolv-  
3           ing the enzyme solution in Tris-HCl buffer solution  
4           containing EDTA, coelenterazine as a substrate and 2-  
5           mercaptoethanol are added to the solution and it is then  
6           allowed to stand on ice, while aequorin is reproduced. A  
7           sample to be analysed is poured into the solution contain-  
8           ing aequorin thus reproduced, and the resulting mixed  
9           solution is transferred to the reaction cell of a spectro-  
10          photometer. A sample to be measured is then injected into  
11          the cell and the quantity of luminescence produced is  
12          measured.

13                 By using this invention, metals can be detected  
14                 by means of aequorin produced in E.coli by the recombinant  
15                 DNA method, using coelenterazine or its analogues as the  
16                 substrate. Therefore, without using natural aequorin,  
17                 which is difficult to obtain, metals can be detected by  
18                 readily available synthetic aequorin produced in E.coli,  
19                 and so can be easily, efficiently and economically detected.

20                 The following non-limitative examples illustrate  
21                 this invention more specifically.

#### EXAMPLE 1

##### Production of aequorin (apoaequorin) from E.coli

##### Construction of piC10:

22                 The plasmid pDR540 having a tac promoter (pro-  
23                 duced by PL Pharmacia Co. Ltd.) was digested by the  
24                 restriction enzyme BamHI-HindIII and then the resulting  
25                 fragment containing the tac promoter of 92bp was separated  
26                 and extracted by the electrophoresis method using acryl-  
27                 amide. Then, the BamHI-HindIII site of the resultant  
28                 vector pUC9 was cloned and piC9 was constructed. Then, in  
29                 order to remove the HindIII site of the expression vector  
30                 piC9, firstly the HindIII was digested and then, after  
31                 repairing the terminals of the vector by E.coli DNA  
32                 polymerase (Klenow fragment) in the presence of dATP,  
33                 dGTP, dCTP and dTTP, both the terminals were ligated with  
34                 dGTP, dCTP and dTTP, both the terminals were ligated with  
35                 dGTP, dCTP and dTTP, both the terminals were ligated with

1     T<sub>4</sub> ligase and piC10 was thus constructed.

EXAMPLE 2

Construction of piC11:

5             In order to bind the aequorin cDNA gene and the  
promoter, synthetic linker nucleotides of AR(5'GATCGATGGTCA-3')  
and AQ(5'AGCTTGACCATC-3') were prepared. After these  
nucleotides were annealed, the 5' terminals were phos-  
phorylated with T<sub>4</sub> nucleotide kinase in the presence of  
ATP. The nucleotides thus phosphorylated were subsequently  
10    cloned, so as to construct in the form of repetition units  
of the linker at the BamHI-EcoRI site of the piC10 (EXAMPLE  
1), and piC11 was constructed.

EXAMPLE 3

Construction of piQ5:

15             The HindIII-EcoRI fragment was separated and  
prepared from aequorin cDNA clone pAQ440, the fragment was  
inserted into the HindIII-EcoRI site of the above piC11,  
and piQ5 was thus obtained. This piQ5 can reproduce  
aequorin protein of the natural type.

20    EXAMPLE 4

Construction of piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE

              After PstI-EcoRI and HindIII-EcoRI fragments  
were separated and purified from cDNA clone pAQ440, these  
fragments were cloned at the restriction enzyme site of  
25    expression vectors pUC9, 9-1, 9-2, having a lac promoter,  
and piQ9PE, 9-1PE, 9-2PE, 9-HE and 9-2HE were constructed.

              All of these piQ fragments were under the control of the  
lac promoter and so comprised aequorin proteins of the  
fused type having 8-amino-acid residues at an N-terminus.

30    EXAMPLE 5

Production of proteins having aequorin activity using  
E.coli:

              The above E.coli strains containing plasmids and  
having 1/100 volumes, obtained by culturing for 12 hours,  
35    were added to 10 ml of LB broth containing 50 µg/ml



1 of ampicillin. The E.coli strains were cultured for 2  
hours at 37°C, subsequently an expression-inducible  
reagent IPTG (isopropyl-β-thiogaractopylanocide) was  
added, to obtain a final concentration of 1 mM, and  
5 incubation was continued for 4 hours at 42°C.

The resultant culture medium was separated by  
centrifugation at 5000 rpm for 10 minutes (Hitachi RP 20)  
and the cells were collected and washed with 5 ml of M9  
salt medium. After resolving the washed cells in 2.5 ml  
10 of 20 mM Tris-HCl buffer (pH 7.6) containing 10 mM of  
EDTA, the cells were destroyed by sonication (60 seconds),  
the mixture was centrifuged at 10,000 rpm for 10 minutes  
and the resulting supernatant was used as an enzyme  
solution for detecting metals.

#### 15 EXAMPLE 6

##### Synthesis of coelenterazine and its analogues

Coelenterazine and its analogues were synthesised  
by the method disclosed by Inoue et al. (Published by  
Japan Chemical Society, Chemistry Letters, 141-144, 1975)  
20 and by the method disclosed by Halt et al. (Biochemistry,  
18, (11) 2204-2210, 1979).

#### EXAMPLE 7

##### Method for detecting metals

The enzyme solution obtained by the above method  
25 was dissolved in 30 mM of Tris-HCl buffer solution contain-  
ing 10 mM of EDTA, 1 μg of the same type of coelenterazine  
substrate as the natural type, obtained by the synthesis  
method disclosed by Inoue et al., and 5 μl of 2-mercapto-  
ethanol were added to the solution to obtain 1 ml of total  
30 volume. The solution was allowed to stand for 1 hour in  
an ice bath and aequorin was reproduced. Then, the  
concentration of metals was measured.

Each sample containing CaCl<sub>2</sub>, SrCl<sub>2</sub> and SmCl<sub>3</sub>  
(1.5 ml, 30 mM) was poured into the solution of aequorin  
35 thus reproduced, the solution was transferred to the

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1 reaction cell of a spectrophotometer (Mitchell-Hasting  
 photometer) and the luminescence of the sample was measured  
 (1). Further, 30 mM of  $\text{CaCl}_2$  was injected into the cell  
 and the luminescence was measured again (2).

5 The results are shown in the following table:

Metal ion	(1)	(2)
$\text{Ca}^{2+}$	8.3	0
$\text{Sr}^{2+}$	4.9	0
$\text{Sm}^{3+}$	3.6	0
$\text{Mn}^{2+}$	0	8.8
$\text{Mg}^{2+}$	0	8.4
$\text{Pb}^{2+}$	0	8.3
$\text{Cd}^{2+}$	0	0.9

20 In the table, the units of the numerals are  $\times 10^{-11}$   
 photo/sec.

It is apparent from the above table that the metal  
 ions  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Sm}^{3+}$  can be detected and the presence  
 of  $\text{Cd}^{2+}$  can be also detected because the inhibitory rate  
 25 of  $\text{Cd}^{2+}$  is 90%.

Metals were detected using synthesised coelenterazine  
 of the natural type in the above examples. Further, when  
 the metals were detected by using analogues of coelenter-  
 azine, similar results were obtained.

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1     CLAIMS:

          1. A method for the detection of metals by  
measuring the luminescence of a metal in the presence of  
5     aequorin, characterised in that the aequorin used has been  
reproduced by adding coelenterazine or an analogue thereof  
to an enzyme of aequorin (apoaequorin).

          2. A method as claimed in claim 1, wherein the  
aequorin enzyme (apoaequorin) is produced in E.coli by a  
10    biosynthesis method.

          3. A method as claimed in claim 2, wherein the  
biosynthesis method is a recombinant DNA technique and the  
aequorin enzyme (apoaequorin) is produced by using aequorin  
cDNA.

15       4. A method as claimed in claim 1, 2 or 3,  
wherein the metal detected is selected from  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  
 $\text{Sm}^{3+}$ ,  $\text{Cd}^{2+}$  and the lanthanoids.

          5. A method as claimed in claim 4, wherein the  
lanthanoids are selected from  $\text{La}^{3+}$ ,  $\text{Tb}^{3+}$  and  $\text{Yb}^{3+}$ .  
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# EUROPEAN SEARCH REPORT

Application number

EP 87 30 4051

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	EP-A-0 137 515 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION) * Pages 9-11 *	1,4	C 12 Q 1/00 C 12 N 15/00
P,X	--- EP-A-0 187 519 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION) * Abstract; page 2, column 1, lines 1-17; page 17, column 2, lines 1-17; pages 29-30 *	2,3	
Y		1,4	
Y	--- TRENDS IN ANALYTICAL CHEMISTRY, vol. 1, no. 16, December 1982, pages 378-383, Elsevier Scientific Publishing Co., Cambridge, GB; F.G. PRENDERGAST: "The use of photoproteins in the detection and quantitation of Ca <sup>2+</sup> in biological systems" * Page 378, column 1, lines 30-33; page 378, column 2, lines 1-45; pages 379-380 *	1,4	
	--- -/-		
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-08-1987	Examiner MEYLAERTS H.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

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# EUROPEAN SEARCH REPORT

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EP 87 30 4051

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	BIOLOGICAL ABSTRACTS, vol. 80, no. 8, 1985, page 474, abstract no. 68408, Philadelphia, Pasadena, US; O. SHIMOMURA et al.: "Halistaurin, phialidin and modified forms of aequorin as calcium indicator in biological systems", & BIOCHEM J. 228(3), 745-750, 1985 * Abstract *	1	
A	--- CHEMICAL ABSTRACTS, vol. 79, no. 13, 1st October 1973, page 185, abstract no. 75180y, Columbus, Ohio, US; O. SHIMOMURA et al.: "Further data on the specificity of aequorin luminescence to calcium", & BIOCHEM. BIOPHYS. RES. COMMUN. 1973, 53(2), 490-4	1,4,5	
P,X	--- BIOCHEMISTRY, vol. 25, no. 26, 30th December 1986, pages 8425-8429, American Chemical Society, Easton, PA, US; S. INOUE et al.: "Expression of apoaquorin complementary DNA in Escherichia coli" * Whole document *	2,3	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-08-1987	Examiner MEYLAERTS H.
<p><b>CATEGORY OF CITED DOCUMENTS</b></p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

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